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TITLE: A peptide from the staphylococcal protein Efb binds P-selectin and inhibits the interaction of platelets with leukocytes

SHORT TITLE: A novel peptide antagonist of P-selectin

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ESSENTIALS

- Efb, an *S. aureus* protein, binds P-selectin and inhibits the platelet/leukocyte interaction.

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- Here, we aimed to identify and characterise the Efb motif responsible for P-selectin binding.
- Efb₆₈₋₈₇ binds platelets in a P-selectin-dependent manner without affecting their activation.
- Efb₆₈₋₈₇ inhibits the formation of PLAs and the platelet-dependent stimulation of NETs *in vitro*.

ABSTRACT

Aims: P-selectin is a key surface adhesion molecule for the interaction of platelets with leukocytes. We have shown previously that the N-terminal domain of *S. aureus* extracellular fibrinogen-binding protein (Efb) binds to P-selectin and interferes with platelet-leukocyte aggregate formation. Here, we aimed to identify the minimal Efb motif required for binding platelets and to characterise its ability to interfering with the formation of platelet-leukocyte aggregates.

Methods and Results: Using a library of synthetic peptides, we mapped the platelet-binding site to a continuous 20 amino acid stretch. The peptide Efb₆₈₋₈₇ was able to bind to resting and, to a greater extent, thrombin-stimulated platelets in the absence of fibrinogen. Dot blots, pull-down assays and P-selectin glycoprotein ligand-1 (PSGL-1) competitive binding experiments identified P-selectin as the cellular docking site mediating Efb₆₈₋₈₇ platelet binding. Accordingly, Efb₆₈₋₈₇ did not bind to other blood cells and captured platelets from human whole blood under low shear stress conditions. Efb₆₈₋₈₇ did not affect platelet activation as tested by aggregometry, flow cytometry and immunoblotting, but inhibited the formation of platelet-leukocyte aggregates (PLAs). Efb₆₈₋₈₇ also interfered with the platelet-dependent stimulation of neutrophil extracellular traps (NETs) formation *in vitro*.

Conclusions: We have identified Efb₆₈₋₈₇ as a novel selective platelet-binding peptide. Efb₆₈₋₈₇ binds directly to P-selectin and inhibits interactions of platelets with leukocytes that lead to PLA and NET formation. As PLAs and NETs play a key role in thromboinflammation, Efb₆₈₋₈₇ is an exciting candidate for the development of novel selective inhibitors of the proinflammatory activity of platelets.

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INTRODUCTION

Our understanding of the role of platelets in vascular health has significantly progressed in recent years. In addition to platelets' established role in haemostasis and thrombosis, their participation in inflammatory responses has become evident (1). The role of platelet pro-inflammatory activity for progression of cardiovascular disease has attracted considerable attention (2). In addition to their ability to release pro-inflammatory cytokines (3) and modulate the release of cytokines by leukocytes (4), platelets directly interact with leukocytes to form platelet-leukocyte aggregates (PLAs) (5). PLA formation has been shown to facilitate leukocyte homing and extravasation at the site of vascular injury, thus promoting inflammation (6). Inflammation is a key factor in vascular complications and cardiovascular diseases (7). PLAs are in fact increased in coronary syndromes, in the form of platelet-monocyte (8-12) and platelet-neutrophil complexes (13). PLA formation also increases as a consequence of coronary surgical intervention (14). Although CD40-CD40L interactions (15) and the binding of the leukocyte receptor CD11b/CD18 (Mac-1) with either platelet glycoprotein receptor GPIB α (16) or platelet integrin $\alpha_{IIb}\beta_3$ (17) participate in the formation of PLAs, the interaction of platelet P-selectin and its physiological ligand P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes is critical for the heterotypic aggregation of these cell types (18). The interaction of platelets with neutrophils has particularly important pathophysiological consequences. Platelets can induce the formation of neutrophil extracellular traps (NETs) (19). While NETs have originally been described in host-defence processes (20), their role in the onset and progression of venous and arterial thrombosis has been shown in animal models and clinical studies (21-23). As for PLAs, the binding of P-selectin on platelets with PSGL-1 on leukocytes is a critical step in NET formation (24).

Because of its role in the interaction of platelets with leukocytes, P-selectin has attracted increasing interest as a drug discovery target to develop pharmacobiological agents able to control inflammation and vascular degeneration via disruption or reduced formation of

PLAs and NETs. The clinical potential of P-selectin inhibitors requires further investigation. We have shown previously that extracellular fibrinogen binding protein (Efb), a protein secreted by *Staphylococcus aureus* (*S. aureus*), directly binds P-selectin and inhibits its interaction with PSGL-1 (25). In contrast to numerous bacterial proteins that have been reported to positively modulate platelet function, Efb inhibits platelet activation and thrombus formation (26, 27), facilitating bacterial survival in the blood and aggravating infection (28). Efb comprises an N-terminal secretion signal, a N-terminal domain lacking structural organisation (Efb-N, residues Ser30-Thr104), and a tri-helical bundle C-terminal domain (Efb-C, residues Ile105-Lys165) (29) (Figure 1A). Efb-N includes two repeated motifs (residues Asn46-Pro67 and Asn77-Ala98) that are homologous to *S. aureus* coagulase repeats and that are part of fibrinogen binding sites comprising residues Ser30-Pro67 and Lys68-Ala98 (30). Efb-C plays an immunosuppressive role by interfering with the complement system (29, 31). In combination, Efb-N and Efb-C facilitate *S. aureus* escape from phagocytosis and increase *S. aureus* pathogenicity (32). In fact, *S. aureus* infections are significantly exacerbated *in vivo* in the presence of Efb (33).

In this study, we mapped the P-selectin-binding site in Efb-N using a peptide scanning approach. A 20 aa-peptide located within Efb-N between Lys68 and Glu87 bound P-selectin. We show here that this peptide selectively binds platelets, without affecting their haemostatic function, and inhibits platelets' ability both to complex with leukocytes to form PLAs and to induce NET formation. The therapeutic potential of this Efb-derived peptide to control thromboinflammation in cardiovascular patients will require further investigation.

METHODS

Peptide synthesis and labelling

All peptides were synthesised by KareBay Bio with the following sequences (purity >98%, Supplementary Figure 1), where (ctrl) designates the scrambled control version:

Efb₃₀₋₄₅ SEGYPREKKPVSIH

Efb₃₀₋₄₅ (ctrl) SPGGKHPVNYKSERIE

Efb₃₀₋₆₇ SEGYPREKKPVSIHNIVEYNDGTFKYQSRPKFNSTP

Efb₃₀₋₆₇ (ctrl) DTYKINSEYPPVRSPNPGGKQYKFSTINGKHEFRVEN

Efb₄₆₋₆₇ NIVEYNDGTFKYQSRPKFNSTP
 Efb₄₆₋₆₇ (ctrl) RKVTPSFYQFNITDKNPGESNY
 Efb₆₈₋₇₆ KYIKFKHDY
 Efb₆₈₋₇₆ (ctrl) KIDKYYHKF
 Efb₆₈₋₈₇ KYIKFKHDYNILEFNDGTFE
 Efb₆₈₋₈₇ (ctrl) YKFEENLFGTNDKDFKIYHI
 Efb₆₈₋₉₉ KYIKFKHDYNILEFNDGTFEYGARPQFNKPAA
 Efb₆₈₋₉₉ (ctrl) KNFELAKAGYPQIAHKTNKPGRFYDDNFIEY
 Efb₇₇₋₉₉ NILEFNDGTFEYGARPQFNKPAA
 Efb₇₇₋₉₉ (ctrl) PFYIAPLARAGTNDNEGFFQENK

In order to fluorescently label lysine residues, synthetic peptides (or recombinant Efb-N₃₀₋₁₀₅) were coupled by direct reaction in 0.1 M sodium carbonate buffer with fluorescein isothiocyanate (FITC). The reaction mixture was incubated, with gentle agitation, at 4°C for 15 hours. Free FITC was eliminated by dialysis in PBS using a 500 Da MWCO cut-off membrane.

Blood collection and platelet isolation

Procedures utilising human blood conformed to the principles outlined in the Declaration of Helsinki. Human blood was collected at the Institute of Clinical Chemistry and Laboratory Medicine (University Medical Center Eppendorf - Hamburg) after informed volunteers' consent was given in written form. Sodium citrate (0.5% w/v) was used as an anticoagulant. Platelet-rich plasma (PRP) was separated from whole blood by centrifugation (250g, 17 minutes) and platelets were separated from PRP by a second centrifugation step (500g, 10 minutes) in the presence of prostaglandin E1 (PGE1, 40 ng/mL) and indomethacin (10 µM). All centrifugations were performed with soft deceleration settings. Platelets were resuspended in modified Tyrode's buffer at a density of 2×10⁸ platelets/ml throughout the study.

Flow cytometry (Efb binding)

For isolated platelet binding, 10⁷ platelets/ml were incubated in the presence or absence of 3 mg/ml fibrinogen and 0.2 U/ml thrombin for 10 minutes at room temperature. 1 µM FITC-Efb peptide, FITC-scrambled Efb peptide or FITC-BSA was then added to the

platelets followed by another 10 minute incubation. Platelet binding was assessed by flow cytometry using a FACSAria III (BD Biosciences).

Alternatively, 10 μ M FITC-conjugated Efb peptide was incubated for 30 minutes at room temperature in heparin-anticoagulated (5 U/ml) human blood with CD42b/PE (#561854, BD Biosciences) and CD45/APC (#555745, BD Biosciences) antibodies. Cells were then fixed with 1% paraformaldehyde (PFA) for 15 minutes and binding was assessed by flow cytometry using a FACSCanto II (BD Biosciences).

Flow cytometry (activation/degranulation markers)

Heparin-anticoagulated (5 U/ml) human blood was preincubated with the relevant peptide for 15 minutes at 37°C. Platelets were activated with 1 U/ml thrombin for 15 minutes at 37°C. Samples were then labelled with anti-CD42b/APC (#551061, BD Biosciences), anti-CD62P/PE (#550561, BD Biosciences), and PAC-1/FITC (#340507, BD Biosciences) antibodies for 15 minutes at room temperature. Samples were then fixed with 4% PFA for 15 minutes at RT. Directly before measurement, the samples were diluted 1:1 with PBS and the fluorescence labelling was measured in 10,000 CD42b/APC-positive events, using a FACSCanto II flow cytometer (BD Biosciences). The mean fluorescence intensity (MFI) of CD42b/APC-positive events in the PE and FITC spectrum bands was analysed.

Isolated platelet fluorescence imaging

Coverslips were coated with 0.01% w/v poly-L-lysine and blocked with 0.1% w/v solution of BSA in PBS. Isolated platelets (resting, or activated in suspension with 10 μ M TRAP6 peptide for 5 minutes at 37°C) were dispensed onto the prepared coverslips at 0.5×10^7 platelets/ml density and incubated for 1h at RT. Platelets were then fixed for 15 minutes in 4% PFA and stained with 2 μ M solution of FITC-labelled peptide (Efb₆₈₋₈₇ or Efb₆₈₋₈₇ (ctrl)) and, subsequently, with 1 U/ml phalloidin-rhodamine in PBS-Tween20 0.1% v/v. The coverslips were mounted onto the glass slides with Fluoromount mounting medium. Imaging was performed with a Leica TCS SP5 confocal microscope.

Dot blots

2 μ g of Efb peptide (Karebay Bio, USA) was dotted onto nitrocellulose membrane and dried. The membrane was then blocked with 5% w/v BSA in TBS-T for 30 minutes before

being incubated in 0.1 ug/ml Fc-P-selectin (R&D Systems, # 137-PS-050) for 45 minutes. Subsequently, anti-P-selectin (Abcam, #ab182135) and IRDye® 800CW Donkey anti-Rabbit IgG (Licor, #926-32213) antibodies were used for staining the membrane. Imaging was performed with a Licor Odyssey CLx scanner.

Pull-down experiments

Efb₆₈₋₈₇ or Efb₆₈₋₈₇ (ctrl) was immobilised on CarboxyLink™ Coupling Resin (Thermo Fisher Scientific), according to the manufacturer's instructions. Peptide-conjugated resin was incubated with platelet lysates prepared by sonication from isolated platelet solution. After binding overnight at 4°C, the peptide complexed to platelet proteins was eluted from the resin according to the manufacturer's instructions. Eluates were subsequently utilised for immunoblotting.

Immunoblotting

Platelet suspensions prepared as described above were stimulated in the presence of 1 mM EGTA and lysed by adding lysis buffer (12.5 mM Tris, pH 8.3, 97 mM glycine, 2% sodium dodecyl sulphate (SDS), 0.5% dithiothreitol (DTT), 10% glycerol, and 0.01% bromophenol blue). Platelet proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane, and analysed by immunoblotting for P-selectin (Abcam, #ab182135), thrombospondin 1 (Thermo Fisher Scientific, #MA5-13398), multimerin 1 (Santa Cruz Biotechnologies, #sc-104427), actin (Merck Millipore #A5441), protein kinase C (PKC) phospho-substrates (#2261, Cell Signaling Technology) and phospho-Src (R&D Systems, # AF2685). Densitometry was performed using ImageJ 1.47v (Wayne Rasband, National Institute of Health, US) and presented as intensity ratio over actin staining (i.e. loading control).

Platelet aggregation

Platelets resuspended in modified Tyrode's buffer at a density of 2×10^8 platelets/ml were stimulated using a Chrono-Log 490 4+4 aggregometer. Aggregation was induced with 0.1 U/ml human thrombin or 3 µg/ml Horm collagen. Absorbance was measured for 10 minutes and expressed as % change of absorbance.

Platelet adhesion/thrombus formation under flow

Vena8-Fluoro+ flow chambers were coated with 0.1mg/ml collagen or Efb₆₈₋₈₇ or Efb₆₈₋₈₇ (ctrl). Blood was taken in 0.25% w/v citrate and 25 μ M D-Phenylalanyl-Prolyl-Arginyl Chloromethyl Ketone (PPACK) and labelled with 1 μ M 3,3'-dihexyloxacarbocyanine iodide (DiOC6) for 10 minutes at 37°C. As indicated in the text, the flow was set to 1000 sec⁻¹ (40 μ l/minute) or 200 sec⁻¹ (8 μ l/minute). Quantification of platelet adhesion after 5 minutes of flow was obtained by LED fluorescence microscopy (EVOS FI) and image analysis using ImageJ 1.47v (Wayne Rasband, National Institute of Health, USA).

PLA quantification

Heparin-anticoagulated (5 U/ml) human blood was treated with red blood cells (RBC) lysis solution (Miltenyi Biotec, Germany) and treated with Efb₆₈₋₈₇ or Efb₆₈₋₈₇ (ctrl) and collagen-related peptide (CRP) (1 μ g/ml), TRAP6 (5 μ M) and/or LPS from *Staphylococcus aureus* (1 μ g/ml) to stimulate platelet-leukocyte aggregation. Anti-CD42b/PE (#561854, BD Biosciences) and anti-CD45/APC (#555745, BD Biosciences) antibodies were used to stain platelets and leukocytes, respectively. Following fixation in 1% w/v PFA, all samples were analysed using a FACSCanto II flow cytometer (BD Biosciences). PLAs were quantified as events positively stained for both markers (see Figure 5A) and their density was expressed as number of double-positive events over 10,000 events.

NET visualisation and quantification

Neutrophils were isolated from human peripheral blood as previously described (34). 5 x 10⁴ neutrophils per well were seeded in 96-well plates and incubated at 37°C for 1 hour to allow cell adhesion. Platelet suspensions (1 x 10⁷ platelets/ml) were incubated for 15 minutes with 5 μ M Efb₆₈₋₈₇ or Efb₆₈₋₈₇ (ctrl) before incubation with TRAP6 (5 μ M)–for 15 minutes to obtain platelet stimulation. Platelet suspensions were then added to the neutrophil monolayers (1 x 10⁶ platelets/well) and incubated for 16 hours to induce NET formation. 0.1 μ M phorbol-12-myristate-13-acetate (PMA) was added to positive control wells. Following fixation with 2% w/v PFA and staining with 1 μ M Sytox Green (Life Technologies), NETs were visualised via fluorescence microscopy (Nikon ECLIPSE

Ts2R with Nikon DS-Fi3 camera) and quantified using a microplate reader (Tecan Spark 10M) using 485 nm_{exc} / 535 nm_{em}.

Statistical analysis

Data normality and homoscedasticity were tested with Shapiro-Wilk and Bartlett's tests, respectively. For dual comparisons (i.e. WT vs 3KO) of normal/homoscedastic data, statistical analysis was performed by unpaired Student's t-tests. Dual comparisons (i.e. WT vs 3KO) of non-normal/non-homoscedastic data were analysed by non-parametric Mann-Whitney test. One-way ANOVA with Bonferroni post-test was used for multiple comparison tests after testing that data are normal and homoscedastic. The software package GraphPad Prism Version 8.1.0 for Windows 64 bit was used for all statistical analyses. For the K_D estimate, we utilised a sigmoidal regression model for one-site saturation binding within GraphPad Prism. Throughout the study, the results were expressed as the mean \pm standard error (SEM). Differences were considered significant at P value < 0.05 (*), 0.01 (**) or 0.001 (***).

RESULTS

Efb₆₈₋₈₇ binds to platelets via P-selectin

Binding experiments identified Efb₆₈₋₈₇ (Figure 1A) as the minimal Efb sequence required to interact with platelets in the absence of fibrinogen (Figure 1B). Platelet stimulation with thrombin increased platelet-binding of Efb₆₈₋₈₇ (Figure 1C). Efb₆₈₋₉₉, a longer peptide including the newly-identified P-selectin-binding motif, displayed the ability to bind platelet upon platelet stimulation but not in unstimulated platelets. The addition of 3 mg/ml fibrinogen (i.e. physiological plasma concentration) stimulated binding of both Efb₆₈₋₈₇ and Efb₃₀₋₆₇ (Figure 1D), although it did not increase the level of Efb₆₈₋₈₇ binding over the value for unstimulated platelets. Fluorescence microscopy (Figure 2A) and flow cytometry (Figure 2B) confirmed binding of FITC-Efb₆₈₋₈₇ to unstimulated and stimulated platelets (TRAP6 and thrombin, respectively). The flow cytometry experiments showed that although the overall amount of bound Efb₆₈₋₈₇ peptide is notably higher after platelet stimulation with thrombin (Figure 2B), the concentration-binding curve indicates a similar K_D for the binding of Efb₆₈₋₈₇ to resting and thrombin-stimulated platelets (5.7 μ M and 3.4 μ M, respectively). The binding site for Efb₆₈₋₈₇ on platelets was investigated by dot blot

(Figure 2C). Fc-P-selectin, a chimeric construct that allows effective suspension of P-selectin in aqueous solution, comprising the extracellular domain of human P-selectin (i.e. residues 42-771) and human IgG1 residues 100-330, was immobilised on nitrocellulose membrane and treated with the library of FITC-labelled Efb peptides. As detected by fluorescence imaging of the membrane, Efb₆₈₋₈₇, Efb₆₈₋₉₉ and Efb-N (i.e. Efb₃₀₋₁₀₅), bound to immobilised P-selectin. All other Efb- and scrambled control-peptides did not exhibit any binding activity to P-selectin. In addition, when immobilised on agarose beads, Efb₆₈₋₈₇ pulled down P-selectin from platelet lysates, whereas it did not show any binding of thrombospondin-1 or multimerin-1, which were previously identified and confirmed as Efb-interacting proteins (25) (Figure 2D). The role of P-selectin in the binding of Efb₆₈₋₈₇ was confirmed by competitive binding experiments using a recombinant chimeric construct of the physiological P-selectin ligand P-selectin glycoprotein ligand- (PSGL-1) (Figure 2E). In these experiments, the binding of FITC-Efb₆₈₋₈₇ was competitively inhibited by Fc-PSGL-1 (R&D Systems, # 3345-PS-050), confirming that P-selectin is the binding site for Efb₆₈₋₈₇ on platelets.

Efb₆₈₋₈₇ selectively binds platelets and captures them from blood under flow

Amongst blood cells, P-selectin is selectively expressed in platelets. Therefore, we assessed whether Efb₆₈₋₈₇ selectively binds platelets or might interact with other blood cells. Antibodies for platelet-specific (CD42b) and leukocyte-specific (CD45) markers were utilised in flow cytometry to identify platelets and leukocytes (white blood cells (WBCs) and red blood cells (RBCs)) (Figure 3A). FITC-Efb₆₈₋₈₇ exclusively bound to platelets, while binding of WBCs and RBCs was negligible. We then used an adhesion assay under physiological flow to probe the stability of Efb₆₈₋₈₇ binding to platelets, assessing whether Efb₆₈₋₈₇ can be used to capture platelets from whole blood. Platelets bound to surfaces coated with Efb₆₈₋₈₇ at venous (200 sec⁻¹) (Figure 3B), but not arterial shear stress (1,000 sec⁻¹) (Supplementary Figure 2). Interestingly, platelet adhesion and thrombus formation on fibrous collagen were not affected by Efb₆₈₋₈₇ (Figure 3C), suggesting that Efb₆₈₋₈₇ does not interfere with platelet function.

Efb₆₈₋₈₇ does not interfere with platelet activation and haemostatic function

We investigated functional implications of Efb₆₈₋₈₇ for platelet function. Platelet aggregation in response to the agonists collagen and thrombin was not affected by platelet pre-treatment with Efb₆₈₋₈₇ compared to Efb₆₈₋₈₇ (ctrl) (Figure 4A). The aggregation in response to secondary agonists ADP and U46619 (a stable analogue of thromboxane A₂) was also unaffected by Efb₆₈₋₈₇ (Supplementary Figure 3). In addition, platelet activation and degranulation were tested by flow cytometry. Data showed that the presence of Efb₆₈₋₈₇ did not significantly change integrin $\alpha_{IIb}\beta_3$ activation (PAC1 antibody) or platelet degranulation (P-selectin externalisation) compared to Efb₆₈₋₈₇ (ctrl) (Figure 4B). Finally, potential effect of Efb₆₈₋₈₇ incubation on intracellular platelets signalling was tested by immunoblotting (Figure 4C). Basal (resting platelets) and activated (thrombin-stimulated platelets) levels of PKC activity and Src kinase activation in the presence of Efb₆₈₋₈₇ and Efb₆₈₋₈₇ (ctrl) were assessed using an anti-phospho PKC substrate antibody (detecting various proteins phosphorylated by classical platelet PKC isoforms α and β) and anti-phospho Src antibody (detecting Src in its active/phosphorylated form). Treatment with Efb₆₈₋₈₇ did not affect activity of these two major platelet signalling pathways. Collectively, these results confirm that Efb₆₈₋₈₇ binding does not interfere with platelet function.

Efb₆₈₋₈₇ inhibits formation of platelet-leukocyte aggregates and reduces formation of neutrophil extracellular traps

The formation of heterotypic cellular complexes between platelets and leukocytes (i.e. platelet-leukocyte aggregates or PLAs) is an important driver of thromboinflammation. Since PLA formation depends on binding of platelet P-selectin to leukocyte PSGL-1, we tested whether Efb₆₈₋₈₇ can block the formation of PLAs in human whole blood. PLAs were detected by flow cytometry as an event highly stained by both anti-CD42b and anti-CD45 antibodies (Figure 5A). PLA levels were significantly increased by stimulation of whole blood with the GPVI receptor agonist CRP, but not the PAR1 agonist TRAP6 or the Toll-like receptor (TLR) agonist LPS used separately (Supplementary Figure 4). The study of Efb₆₈₋₈₇'s effect on PLA formation was performed with combined stimulation by CRP, TRAP6 and LPS. Substantial inhibition of PLA formation was observed at 1 μ M or higher concentrations of Efb₆₈₋₈₇ (Figure 5B). Since NET formation is another process dependent on P-selectin binding to PSGL-1 (24), we next investigated whether Efb₆₈₋₈₇

interferes with the stimulation of NET formation by platelets. Neutrophils isolated from human peripheral blood were allowed to adhere and were then treated with platelets. Resting platelets were compared to platelets treated with TRAP6 (5 μ M). Stimulated platelets were able to induce a significant formation of NETs (Figure 6), which was inhibited by Efb₆₈₋₈₇, but not with the control peptide for Efb₆₈₋₈₇. The incubation of neutrophils with the above agonists in the absence of platelets did not lead to formation of NETs. Direct stimulation of NETs by neutrophil treatment with PMA was used as positive control, which was not affected by Efb₆₈₋₈₇ (data not shown).

DISCUSSION

Discovery of their involvement in inflammatory responses and vascular degeneration has added an important new chapter in our understanding of the role of platelets in health and disease. In addition to the release of pro-inflammatory cytokines (35), the function of platelets in inflammatory processes involves the formation of PLAs, which mediate homing and extravasation of leukocytes at the site of vascular damage (6, 36). The interaction of P-selectin with PSGL-1 is the key molecular event for PLA formation (4). Other key surface receptors involved in PLA formation are activated in response to P-selectin-PSGL-1 interaction. The activation of the leukocyte integrin $\alpha_M\beta_2$ (macrophage-1 antigen or MAC-1) by P-selectin-PSGL-1 interaction, for example, is a key step in the formation of heterotypic complexes between platelets and leukocytes (37). In addition to linking inflammation with platelet activation and thrombus formation, and supporting the concept of thromboinflammation (38), the formation of PLAs has far-reaching consequences for cardiovascular health. Formation of heterotypic complexes between platelets and monocytes regulates the coagulation cascade through the upregulation of tissue factor (39), which exacerbates the thrombotic risk for patients with a variety of conditions, including COVID-19 (40).

The P-selectin/PSGL-1 axis is therefore an attractive target for the development of novel antiinflammatory and vasculoprotective drugs. Although some reduction of PLAs can be achieved by traditional antiplatelet therapy (41), it is important to find strategies for achieving more comprehensive abatement of PLAs and avoiding the bleeding risk associated with existing antithrombotics. This makes the development of a safe and efficacious P-selectin antagonist an attractive therapeutic option in cardiovascular

medicine. Although several P-selectin antagonists have been described and proposed as candidates for drug development, there is currently no P-selectin inhibitor validated for clinical use. Recombinantly expressed vimentin (42), synthetic glycomimetics of the N-terminus of PSGL-1 (43) and anti-P-selectin antibodies (44) have been characterised for their ability to compete with PLA formation *in vitro* and *in vivo*, while a promising small molecule failed to show any effect *in vivo* (45). The cost and pharmacokinetic properties of full recombinant proteins, large post-translationally modified peptides or antibodies make the search for novel candidate antagonists for P-selectin a relevant, timely and unresolved challenge for vascular drug discovery. In this study, we therefore followed a different approach. Based on our previous work showing that the bacterial protein Efb binds directly to P-selectin, inhibits its interaction with PSGL-1 and interferes with PLA formation (25), we set out to identify an Efb-derived peptide that retains the ability to bind P-selectin and inhibit PLA formation.

The peptide we identified is Efb₆₈₋₈₇, a twenty amino acid sequence from the N-terminal domain of Efb and part of one of two previously identified Efb binding sites for fibrinogen (30). Efb₆₈₋₈₇ binding to platelets is not increased by addition of exogenous fibrinogen and is therefore fibrinogen-independent (Figure 1D). On the other hand, Efb₃₀₋₆₇ interacts with platelets only in the presence of exogenous fibrinogen. Efb₃₀₋₆₇ corresponds to the other previously identified fibrinogen binding site (30). Interestingly, Efb₆₈₋₈₇ shows similar binding affinity to resting and stimulated platelets, but the Efb₆₈₋₈₇ binding capacity of platelets (i.e. amount of peptide bound per platelet) is significantly increased by stimulation (Figures 1B and 1C). This binding profile is compatible with a Binding site that is present at a low level on the surface of resting platelets but that undergoes an activation-dependent increase in level. This is characteristic of P-selectin levels which increase on the surface of platelets as a consequence of stimulation-dependent degranulation (i.e. migration to the cell periphery and fusion with the plasma membrane of P-selectin-rich alpha granules) (46). These data were supported by dot blot experiments, which confirmed direct binding of Efb₆₈₋₈₇ to P-selectin (Figure 2C). Efb₆₈₋₉₉ was also able to bind to P-selectin in the dot blot experiments, although the level of staining was visibly lower. Further experiments would be required to assess whether the residues between Tyr88 and Ala99 may interfere with the binding of P-selectin. It is also noteworthy that contrarily to Efb₃₀₋₁₀₅ (also known as Efb-N) (25), Efb₆₈₋₈₇ does not interact

with multimerin-1 and thrombospondin-1. Since the interaction of Efb-N with multimerin-1 and thrombospondin-1 was detected with the same approach utilised in this study (i.e. peptide conjugation and platelet protein pull-down), the most likely explanation is that the binding sites for these proteins map outside the Lys68-Glu87 portion of Efb-N. This observation emphasises the modular and multifunctional nature of Efb, with separate or overlapping binding sites for multiple target proteins. The modular multifunctionality of Efb could facilitate further biotechnological applications based on the use of this protein.

Efb₆₈₋₈₇ represents a promising candidate for the development of a novel pharmacological agent with antiinflammatory or antithrombotic properties based on antagonism of P-selectin and inhibition of PLA formation. This approach would have the advantage of leaving unaffected the haemostatic function of platelets, which should reduce or abolish any bleeding side effects. The use of current antiplatelet drugs from acetylsalicylic acid (ASA) to P2Y₁₂ antagonists (such as Clopidogrel, Prasugrel and Ticagrelor) to integrin inhibitors (such as Abciximab or Tirofiban) is associated with an increase in haemorrhagic risk for patients (47, 48). This is likely to be the consequence of targeting a molecular pathway normally required for haemostasis (i.e. cyclooxygenases, ADP receptors or integrins). By targeting a surface adhesion molecule such as P-selectin that is not involved in healthy haemostatic responses, Efb₆₈₋₈₇ would selectively diminish or abolish the contribution of platelets to the onset and/or progression of thromboinflammation, with little or no risk of causing haemorrhagic complications.

We demonstrated in addition that Efb₆₈₋₈₇ does not bind to other blood cells (Figure 3A) and that Efb₆₈₋₈₇ does not interfere with the activation and function of platelets in response to physiological stimuli (Figure 4). Efb₆₈₋₈₇ can therefore be used as a tool to selectively label platelets without affecting their responsiveness, which could find application in research and diagnostic laboratory practice. The ability to sequester platelets from whole blood (Figure 3B) could be investigated further to develop a novel platelet isolation method. Since Efb₆₈₋₈₇ displayed increased binding to activated platelets (Figure 2B), it may be possible to develop a method to selectively capture activated platelets from whole blood. The selective reduction of the count of circulating activated platelets could find clinical application because an increase in circulating activated platelets is observed in several cardiovascular diseases and is proposed as a disease mechanism leading to thrombosis (49, 50).

In summary, we present here the identification and validation of Efb₆₈₋₈₇, a novel P-selectin antagonist derived from the N-terminal domain of the *S. aureus* protein Efb. In addition to confirming the selective binding to P-selectin, we have established the ability of Efb₆₈₋₈₇ to interfere with PLA and platelet-induced NET formation *in vitro* without affecting platelet signalling and platelet functional responses. As the formation of PLAs and NETs is critical for the progression of vascular inflammation and its association with thrombotic complications, Efb₆₈₋₈₇ has the potential to become a clinical tool for the treatment of conditions ranging from major blood vessel atherosclerosis to microcirculatory dysfunction. Future clinical studies on this peptide may lead to the development of a novel treatment to help in the battle against thromboinflammatory conditions.

Author contribution statement

SW, NW, HE, MP and AU performed experiments and part of the data analysis for this manuscript (the order reflects the level of involvement in this project). TR, IE and SB provided critical revisions of the manuscript. SB and GP designed the project and planned the experiments. GP wrote the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

Data availability statement

Data in this article will be shared upon reasonable requests to the corresponding author by email.

FIGURE LEGENDS

Figure 1. Synthetic Efb peptides bind human platelets. (A) Structure of Efb and the design of synthetic peptides representing different regions of the Efb N-terminal domain (amino acids 30-105). The signal peptide is shown in light blue, the first fibrinogen binding motif (Fg B1) in red, the second fibrinogen binding motif (Fg B2) in green. Efb peptide binding to resting (B), thrombin-stimulated (C) or fibrinogen-treated (D) platelets. 1 μ M FITC-labelled Efb or scrambled control peptides were incubated with isolated human platelets and their binding was assessed by flow cytometry. In C), thrombin stimulation was obtained with 0.1 u/ml human thrombin for 10 minutes, while in D) platelets were treated with 3 mg/ml fibrinogen for 10 minutes. Values are the median fluorescence intensity (MFI) from 10,000 events in five independent experiments (mean \pm SEM). Statistical significance was assessed using one-way ANOVA with Bonferroni post-hoc test; P-value < 0.05 (*), P-value < 0.01 (**), P-value < 0.001 (***).

Figure 2. Efb₆₈₋₈₇ binding to platelet P-selectin. (A) Phalloidin-rhodamine (red, 1 U/ml) and FITC-labelled Efb₆₈₋₈₇ (green, 2 μ M) were used to stain human platelets fixed either before or after stimulation in suspension with TRAP6. FITC-labelled scrambled Efb₆₈₋₈₇ was used as control. Images are representative of five independent experiments. The scale bar indicated in white is 10 μ m. (B) Using different concentrations of FITC-Efb₆₈₋₈₇ (0.1 – 100 μ M), Efb₆₈₋₈₇ binding affinity to resting and thrombin-stimulated platelets was assessed by flow cytometry. Data are % of the maximal binding values and are mean \pm SEM from five independent experiments. (C) The direct interaction of Efb₆₈₋₈₇ and P-selectin was confirmed by dot blot. Fc-P-selectin was dotted onto individual nitrocellulose membranes and allowed to dry. After blocking with 5% w/v BSA, each FITC-Efb and FITC-scrambled Efb peptide (0.3 μ g/ml of 68-87) was incubated on the membrane. Fluorescence images of the membranes were obtained with a ChemiDoc Scanner (Bio-Rad) at 488 nm_{exc}. The data are representative of six independent measurements. (D) Pull-down experiments performed with Efb₆₈₋₈₇ conjugated to agarose beads confirmed P-selectin as the main candidate binding site for this peptide on platelets. Platelet lysate (lane 1), pull-downs with unconjugated beads (lane 2), pull-downs with beads conjugated with scrambled Efb₆₈₋₈₇ (lane 3) and pull-downs with beads conjugated with Efb₆₈₋₈₇ (lane 4) were compared. The immunoblotting was performed with antibodies for human P-

selectin, multimerin-1 or thrombospondin-1. The data are representative of five independent experiments. (E) Binding competition by Fc-PSGL-1. As described for (B), the binding of FITC-Efb₆₈₋₈₇ to platelets was assessed by flow cytometry in the presence of different concentrations of Fc-PSGL-1 (0, 10 or 30 μ M). Values are the median fluorescence intensity (MFI) from 10,000 events. Statistical significance was assessed using one-way ANOVA with Bonferroni post-hoc test: P-value < 0.05 (*), P-value < 0.01 (**), P-value < 0.001 (***).

Figure 3. Efb₆₈₋₈₇ binds to platelets in whole blood without interfering with collagen-dependent thrombus formation. (A) CD42b/PE and CD45-APC antibodies were used to distinguish platelets (CD42b⁺), leukocytes (CD42b⁻, CD45⁺) and red blood cells (CD42b⁻, CD45⁻) from human blood by flow cytometry. FITC-Efb₆₈₋₈₇ binding to each cell population was tested by flow cytometry. Data are mean \pm SEM of the MFI values from 10,000 events in 6 independent experiments. Statistical significance was assessed using one-way ANOVA with Bonferroni post-hoc test: P-value < 0.05 (*), P-value < 0.01 (**), P-value < 0.001 (***). (B) Platelet adhesion to Efb₆₈₋₈₇ under physiological flow. Platelet adhesion to Efb₆₈₋₈₇ was tested at shear stress 200 sec⁻¹ (venous) and 1,000 sec⁻¹ (arterial, not shown). In addition, (C) the effect of Efb₆₈₋₈₇ (5 μ M) on platelet adhesion and thrombus formation on collagen was tested (1,000 sec⁻¹). Platelets in whole blood were fluorescently labelled with DiOC₆ and their surface coverage was assessed by fluorescence imaging/densitometry analysis. Data presented are mean \pm SEM of the surface coverage from 6 independent experiments. Statistical significance was assessed using the paired sample Student t-test (normality was assessed by Shapiro-Wilk test): P-value < 0.05 (*), P-value < 0.01 (**), P-value < 0.001 (***).

Figure 4. Efb₆₈₋₈₇ does not interfere with the normal functions of platelets. (A) Platelet aggregation induced by thrombin (0.1 U/ml) and collagen (10 μ g/ml) was tested in the presence of 5 μ M Efb₆₈₋₈₇ or scrambled Efb₆₈₋₈₇. Representative examples of aggregations from 4 independent experiments are shown and a bar graph shows mean \pm SEM. No statistical significance was detected using the paired sample Student t-test. (B) Flow cytometry analysis of platelet activation and degranulation. Resting and thrombin-stimulated (1 U/ml) platelets were compared for degranulation (anti-CD42b/APC) and

integrin $\alpha_{IIb}\beta_3$ activation (anti-PAC-1/FITC) in the presence of 5 μ M Efb₆₈₋₈₇ or scrambled Efb₆₈₋₈₇. A representative forward scattering (FSC) / side scattering (SSC) dot plot of isolated platelets in the presence of Efb₆₈₋₈₇ at resting (blue) and thrombin-stimulated (red) is also shown. The statistical significance between Efb₆₈₋₈₇ and the control peptide was assessed using one-way ANOVA with Bonferroni post-test; P-value < 0.01 (**), P-value < 0.001 (***), error bars represent mean \pm SEM from 4 independent experiments. (C) Platelet signalling was studied by immunoblotting in the presence of Efb₆₈₋₈₇ or Efb₆₈₋₈₇ ctrl peptides (10 μ M). Platelets were activated with 0.1 U/ml thrombin (in the presence of 1mM EGTA to avoid platelet aggregation and so facilitate protein extraction). Following lysis and SDS-PAGE, lysates were immunoblotted for protein kinase C (PKC) phosphorylated substrates of classical protein kinase C (PKC) isoforms, phosphorylated Src and actin. Data were quantified by densitometry using ImageJ 1.47v, presented as intensity ratio over actin staining (i.e. loading control) and are mean \pm SEM from five independent experiments. No statistical significance was detected using a paired sample Student t-test (normality was assessed by Shapiro-Wilk test).

Figure 5. Efb₆₈₋₈₇ inhibits the formation of PLAs in human whole blood. (A) Human blood was stimulated with CRP (1 μ g/ml), TRAP6 (5 μ M) and LPS from *Staphylococcus aureus* (1 μ g/ml) for 30 minutes. Anti-CD42b/PE and anti-CD45/APC antibodies were used to stain platelets and leukocytes. PLAs were quantified in the top right quadrant of the PE/APC plot. (B) Where indicated, 0.1 - 100 μ M Efb₆₈₋₈₇ (or control peptide) was co-incubated with the agonists. Data are the number of PLA per 10,000 events (i.e. blood cells) and represent mean \pm SEM from five independent experiments. Statistical significance was assessed using one-way ANOVA with Bonferroni post-hoc test: P-value < 0.05 (*), P-value < 0.01 (**), P-value < 0.001 (***).

Figure 6. Efb₆₈₋₈₇ inhibits the formation of platelet-induced NETs. 5 x 10⁴/well neutrophils were seeded into 96-well plates and incubated at 37 °C for 1 hour. 5 μ M Efb₆₈₋₈₇, scrambled control peptide and vehicle solution were added to platelets before stimulation with TRAP6 (5 M). 1 x 10⁶ platelets were added to each well. Negative control wells were prepared by adding either only neutrophils or only platelets with TRAP6 (5 M). The positive control wells are neutrophils treated with 0.1 μ M PMA. The plates were

incubated for 16 hours at 37 °C with 5% CO₂, before fixation in PFA and staining of NETs with Sytox Green. (A) Representative images of selected conditions from five independent experiments were obtained by fluorescence microscopy and are shown in the top panels. (B) The quantification of NET release was obtained by staining of the cell supernatants by fluorescence microplate reading after addition of Sytox green. Data are the mean \pm SEM from five independent experiments. Statistical significance was assessed using one-way ANOVA with Bonferroni post-hoc test: P-value < 0.05 (*), P-value < 0.01 (**), P-value < 0.001 (***).

REFERENCES

1. Kubes, P. (2016) The versatile platelet contributes to inflammation, infection, hemostasis, coagulation and cancer. *Semin Immunol* **28**, 535
2. Koupenova, M., Clancy, L., Corkrey, H. A., and Freedman, J. E. (2018) Circulating Platelets as Mediators of Immunity, Inflammation, and Thrombosis. *Circ Res* **122**, 337-351
3. Thomas, M. R., and Storey, R. F. (2015) The role of platelets in inflammation. *Thromb Haemost* **114**, 449-458
4. Dib, P. R. B., Quirino-Teixeira, A. C., Merij, L. B., Pinheiro, M. B. M., Rozini, S. V., Andrade, F. B., and Hottz, E. D. (2020) Innate immune receptors in platelets and platelet-leukocyte interactions. *J Leukoc Biol* **108**, 1157-1182
5. Schrottmaier, W. C., Mussbacher, M., Salzmann, M., and Assinger, A. (2020) Platelet-leukocyte interplay during vascular disease. *Atherosclerosis* **307**, 109-120
6. Diacovo, T. G., Roth, S. J., Buccola, J. M., Bainton, D. F., and Springer, T. A. (1996) Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood* **88**, 146-157
7. Ruparelina, N., Chai, J. T., Fisher, E. A., and Choudhury, R. P. (2017) Inflammatory processes in cardiovascular disease: a route to targeted therapies. *Nat Rev Cardiol* **14**, 133-144
8. Allen, N., Barrett, T. J., Guo, Y., Nardi, M., Ramkhalawon, B., Rockman, C. B., Hochman, J. S., and Berger, J. S. (2019) Circulating monocyte-platelet aggregates

- are a robust marker of platelet activity in cardiovascular disease. *Atherosclerosis* **282**, 11-18
9. Brown, R. A., Lip, G. Y. H., Varma, C., and Shantsila, E. (2018) Impact of Mon2 monocyte-platelet aggregates on human coronary artery disease. *Eur J Clin Invest* **48**, e12911
 10. Czepluch, F. S., Kuschicke, H., Dellas, C., Riggert, J., Hasenfuss, G., and Schafer, K. (2014) Increased proatherogenic monocyte-platelet cross-talk in monocyte subpopulations of patients with stable coronary artery disease. *J Intern Med* **275**, 144-154
 11. Wrigley, B. J., Shantsila, E., Tapp, L. D., and Lip, G. Y. (2013) Increased formation of monocyte-platelet aggregates in ischemic heart failure. *Circ Heart Fail* **6**, 127-135
 12. Sarma, J., Laan, C. A., Alam, S., Jha, A., Fox, K. A., and Dransfield, I. (2002) Increased platelet binding to circulating monocytes in acute coronary syndromes. *Circulation* **105**, 2166-2171
 13. Nijm, J., Wikby, A., Tompa, A., Olsson, A. G., and Jonasson, L. (2005) Circulating levels of proinflammatory cytokines and neutrophil-platelet aggregates in patients with coronary artery disease. *Am J Cardiol* **95**, 452-456
 14. Ivert, T., Dalen, M., Ander, C., Stalesen, R., Lordkipanidze, M., and Hjendahl, P. (2019) Increased platelet reactivity and platelet-leukocyte aggregation after elective coronary bypass surgery. *Platelets* **30**, 975-981
 15. Willecke, F., Tiwari, S., Rupprecht, B., Wolf, D., Hergeth, S., Hoppe, N., Dufner, B., Schulte, L., Anto Michel, N., Bukosza, N., Marchini, T., Jackel, M., Stachon, P., Hilgendorf, I., Zeschky, K., Schleicher, R., Langer, H. F., von Zur Muhlen, C., Bode, C., Peter, K., and Zirlik, A. (2014) Interruption of classic CD40L-CD40 signalling but not of the novel CD40L-Mac-1 interaction limits arterial neointima formation in mice. *Thromb Haemost* **112**, 379-389
 16. Simon, D. I., Chen, Z., Xu, H., Li, C. Q., Dong, J., McIntire, L. V., Ballantyne, C. M., Zhang, L., Furman, M. I., Berndt, M. C., and Lopez, J. A. (2000) Platelet glycoprotein Iba1 is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J Exp Med* **192**, 193-204

17. Patko, Z., Csaszar, A., Acsady, G., Peter, K., and Schwarz, M. (2012) Roles of Mac-1 and glycoprotein IIb/IIIa integrins in leukocyte-platelet aggregate formation: stabilization by Mac-1 and inhibition by GpIIb/IIIa blockers. *Platelets* **23**, 368-375
18. Yokoyama, S., Ikeda, H., Haramaki, N., Yasukawa, H., Murohara, T., and Imaizumi, T. (2005) Platelet P-selectin plays an important role in arterial thrombogenesis by forming large stable platelet-leukocyte aggregates. *J Am Coll Cardiol* **45**, 1280-1286
19. Caudrillier, A., Kessenbrock, K., Gilliss, B. M., Nguyen, J. X., Marques, M. B., Monestier, M., Toy, P., Werb, Z., and Looney, M. R. (2012) Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J Clin Invest* **122**, 2661-2671
20. Fuchs, T. A., Brill, A., and Wagner, D. D. (2012) Neutrophil extracellular trap (NET) impact on deep vein thrombosis. *Arterioscler Thromb Vasc Biol* **32**, 1777-1783
21. Fuchs, T. A., Brill, A., Duerschmied, D., Schatzberg, D., Monestier, M., Myers, D. D., Jr., Wroblewski, S. K., Wakefield, T. W., Hartwig, J. H., and Wagner, D. D. (2010) Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci U S A* **107**, 15880-15885
22. Farkas, A. Z., Farkas, V. J., Gubucz, I., Szabo, L., Balint, K., Tenekedjiev, K., Nagy, A. I., Sotonyi, P., Hidi, L., Nagy, Z., Szikora, I., Merkely, B., and Kolev, K. (2019) Neutrophil extracellular traps in thrombi retrieved during interventional treatment of ischemic arterial diseases. *Thromb Res* **175**, 46-52
23. Sharma, S., Hofbauer, T. M., Ondracek, A. S., Chausheva, S., Alimohammadi, A., Artner, T., Panzenboeck, A., Rinderer, J., Shafran, I., Mangold, A., Winker, R., Wohlschlager-Krenn, E., Moser, B., Taghavi, S., Klepetko, W., Preissner, K. T., and Lang, I. M. (2021) Neutrophil extracellular traps promote fibrous vascular occlusions in chronic thrombosis. *Blood* **137**, 1104-1116
24. Etulain, J., Martinod, K., Wong, S. L., Cifuni, S. M., Schattner, M., and Wagner, D. D. (2015) P-selectin promotes neutrophil extracellular trap formation in mice. *Blood* **126**, 242-246
25. Posner, M. G., Upadhyay, A., Abubaker, A. A., Fortunato, T. M., Vara, D., Canobbio, I., Bagby, S., and Pula, G. (2016) Extracellular Fibrinogen-binding

- Protein (Efb) from *Staphylococcus aureus* Inhibits the Formation of Platelet-Leukocyte Complexes. *J Biol Chem* **291**, 2764-2776
26. Shannon, O., Uekotter, A., and Flock, J. I. (2005) Extracellular fibrinogen binding protein, Efb, from *Staphylococcus aureus* as an antiplatelet agent in vivo. *Thromb Haemost* **93**, 927-931
27. Shannon, O., and Flock, J. I. (2004) Extracellular fibrinogen binding protein, Efb, from *Staphylococcus aureus* binds to platelets and inhibits platelet aggregation. *Thromb Haemost* **91**, 779-789
28. Zhang, X., Liu, Y., Gao, Y., Dong, J., Mu, C., Lu, Q., Shao, N., and Yang, G. (2011) Inhibiting platelets aggregation could aggravate the acute infection caused by *Staphylococcus aureus*. *Platelets* **22**, 228-236
29. Hammel, M., Sfyroera, G., Ricklin, D., Magotti, P., Lambris, J. D., and Geisbrecht, B. V. (2007) A structural basis for complement inhibition by *Staphylococcus aureus*. *Nat Immunol* **8**, 430-437
30. Ko, Y. P., Liang, X., Smith, C. W., Degen, J. L., and Hook, M. (2011) Binding of Efb from *Staphylococcus aureus* to fibrinogen blocks neutrophil adherence. *J Biol Chem* **286**, 9865-9874
31. Lee, L. Y., Hook, M., Haviland, D., Wetsel, R. A., Yonter, E. O., Syribeys, P., Vernachio, J., and Brown, E. L. (2004) Inhibition of complement activation by a secreted *Staphylococcus aureus* protein. *J Infect Dis* **190**, 571-579
32. Ko, Y. P., Kuipers, A., Freitag, C. M., Jongerius, I., Medina, E., van Rooijen, W. J., Spaan, A. N., van Kessel, K. P., Hook, M., and Rooijackers, S. H. (2013) Phagocytosis escape by a *Staphylococcus aureus* protein that connects complement and coagulation proteins at the bacterial surface. *PLoS Pathog* **9**, e1003816
33. Jongerius, I., von Kockritz-Blickwede, M., Horsburgh, M. J., Ruyken, M., Nizet, V., and Rooijackers, S. H. (2012) *Staphylococcus aureus* virulence is enhanced by secreted factors that block innate immune defenses. *J Innate Immun* **4**, 301-311
34. Brinkmann, V., Laube, B., Abu Abed, U., Goosmann, C., and Zychlinsky, A. (2010) Neutrophil extracellular traps: how to generate and visualize them. *J Vis Exp*
35. Bakogiannis, C., Sachse, M., Stamatelopoulos, K., and Stellos, K. (2019) Platelet-derived chemokines in inflammation and atherosclerosis. *Cytokine* **122**, 154157

36. Diacovo, T. G., Puri, K. D., Warnock, R. A., Springer, T. A., and von Andrian, U. H. (1996) Platelet-mediated lymphocyte delivery to high endothelial venules. *Science* **273**, 252-255
37. Wang, H. B., Wang, J. T., Zhang, L., Geng, Z. H., Xu, W. L., Xu, T., Huo, Y., Zhu, X., Plow, E. F., Chen, M., and Geng, J. G. (2007) P-selectin primes leukocyte integrin activation during inflammation. *Nat Immunol* **8**, 882-892
38. Totani, L., and Evangelista, V. (2010) Platelet-leukocyte interactions in cardiovascular disease and beyond. *Arterioscler Thromb Vasc Biol* **30**, 2357-2361
39. Steiner, S., Seidinger, D., Huber, K., Kaun, C., Minar, E., and Kopp, C. W. (2003) Effect of glycoprotein IIb/IIIa antagonist abciximab on monocyte-platelet aggregates and tissue factor expression. *Arterioscler Thromb Vasc Biol* **23**, 1697-1702
40. Hottz, E. D., Azevedo-Quintanilha, I. G., Palhinha, L., Teixeira, L., Barreto, E. A., Pao, C. R. R., Righy, C., Franco, S., Souza, T. M. L., Kurtz, P., Bozza, F. A., and Bozza, P. T. (2020) Platelet activation and platelet-monocyte aggregate formation trigger tissue factor expression in patients with severe COVID-19. *Blood* **136**, 1330-1341
41. Cao, Y. J., Wang, Y. M., Zhang, J., Zeng, Y. J., and Liu, C. F. (2009) The effects of antiplatelet agents on platelet-leukocyte aggregations in patients with acute cerebral infarction. *J Thromb Thrombolysis* **27**, 233-238
42. Lam, F. W., Da, Q., Guillory, B., and Cruz, M. A. (2018) Recombinant Human Vimentin Binds to P-Selectin and Blocks Neutrophil Capture and Rolling on Platelets and Endothelium. *J Immunol* **200**, 1718-1726
43. Chaikof, E. L., Wong, D. J., Park, D. D., Park, S. S., Haller, C., Chen, J., Dai, E., Liu, L., Mandhapat, A. R., Eradi, P., Dhakal, B., Wever, W., Hanes, M., Sun, L., and Cummings, R. D. (2021) A PSGL-1 Glycomimetic Reduces Thrombus Burden Without Affecting Hemostasis. *Blood*
44. Kling, D., Stucki, C., Kronenberg, S., Tuerck, D., Rheume, E., Tardif, J. C., Gaudreault, J., and Schmitt, C. (2013) Pharmacological control of platelet-leukocyte interactions by the human anti-P-selectin antibody inclacumab--preclinical and clinical studies. *Thromb Res* **131**, 401-410

45. Japp, A. G., Chelliah, R., Tattersall, L., Lang, N. N., Meng, X., Weisel, K., Katz, A., Burt, D., Fox, K. A., Feuerstein, G. Z., Connolly, T. M., and Newby, D. E. (2013) Effect of PSI-697, a novel P-selectin inhibitor, on platelet-monocyte aggregate formation in humans. *J Am Heart Assoc* **2**, e006007
46. Vandendries, E. R., Furie, B. C., and Furie, B. (2004) Role of P-selectin and PSGL-1 in coagulation and thrombosis. *Thromb Haemost* **92**, 459-466
47. Palmerini, T., Bacchi Reggiani, L., Della Riva, D., Romanello, M., Feres, F., Abizaid, A., Gilard, M., Morice, M. C., Valgimigli, M., Hong, M. K., Kim, B. K., Jang, Y., Kim, H. S., Park, K. W., Colombo, A., Chieffo, A., Ahn, J. M., Park, S. J., Schupke, S., Kastrati, A., Montalescot, G., Steg, P. G., Diallo, A., Vicaut, E., Helft, G., Biondi-Zoccai, G., Xu, B., Han, Y., Genereux, P., Bhatt, D. L., and Stone, G. W. (2017) Bleeding-Related Deaths in Relation to the Duration of Dual-Antiplatelet Therapy After Coronary Stenting. *J Am Coll Cardiol* **69**, 2011-2022
48. Costa, F., Van Klaveren, D., Feres, F., James, S., Raber, L., Pilgrim, T., Hong, M. K., Kim, H. S., Colombo, A., Steg, P. G., Bhatt, D. L., Stone, G. W., Windecker, S., Steyerberg, E. W., Valgimigli, M., and Investigators, P.-D. S. (2019) Dual Antiplatelet Therapy Duration Based on Ischemic and Bleeding Risks After Coronary Stenting. *J Am Coll Cardiol* **73**, 741-754
49. Xu, Y., Huo, Y., Toufektsian, M. C., Ramos, S. I., Ma, Y., Tejani, A. D., French, B. A., and Yang, Z. (2006) Activated platelets contribute importantly to myocardial reperfusion injury. *Am J Physiol Heart Circ Physiol* **290**, H692-699
50. Huo, Y., Schober, A., Forlow, S. B., Smith, D. F., Hyman, M. C., Jung, S., Littman, D. R., Weber, C., and Ley, K. (2003) Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med* **9**, 61-67

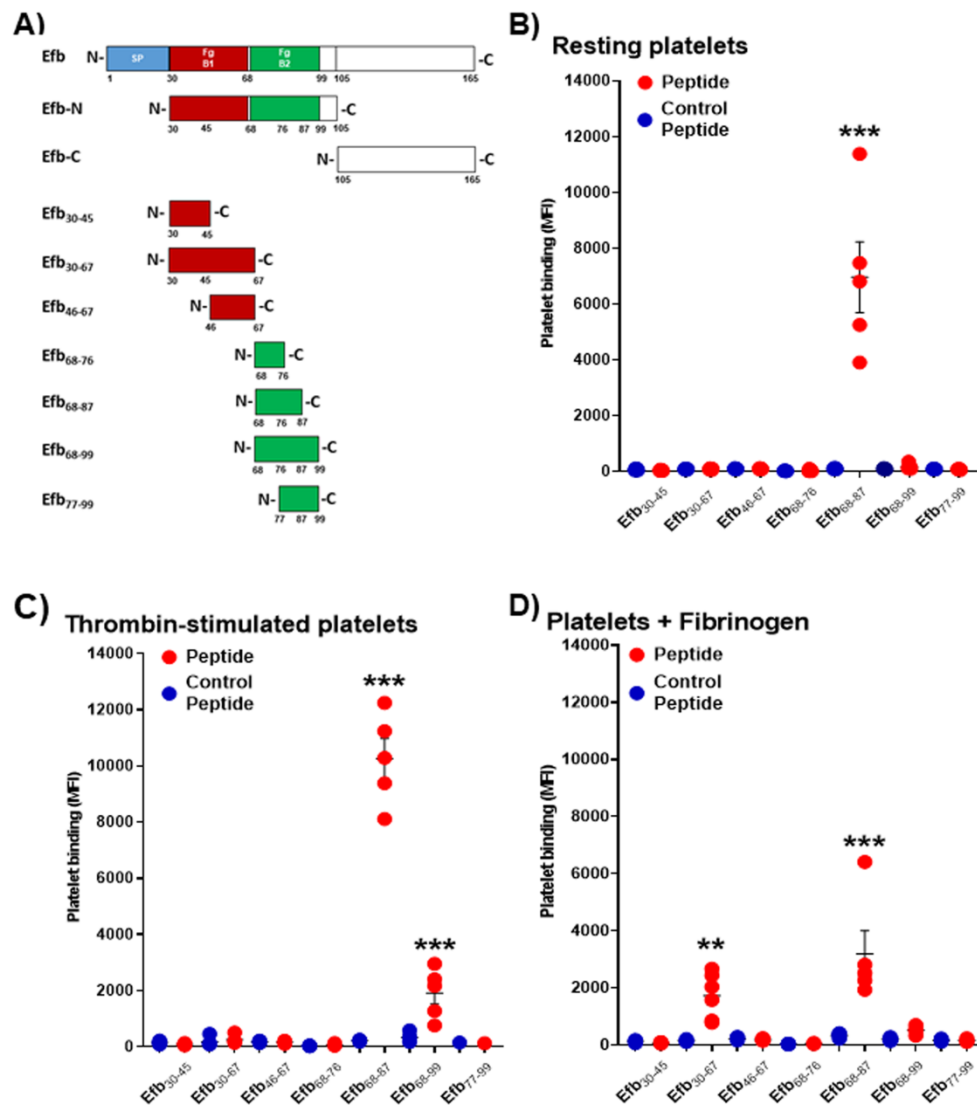


Figure 1

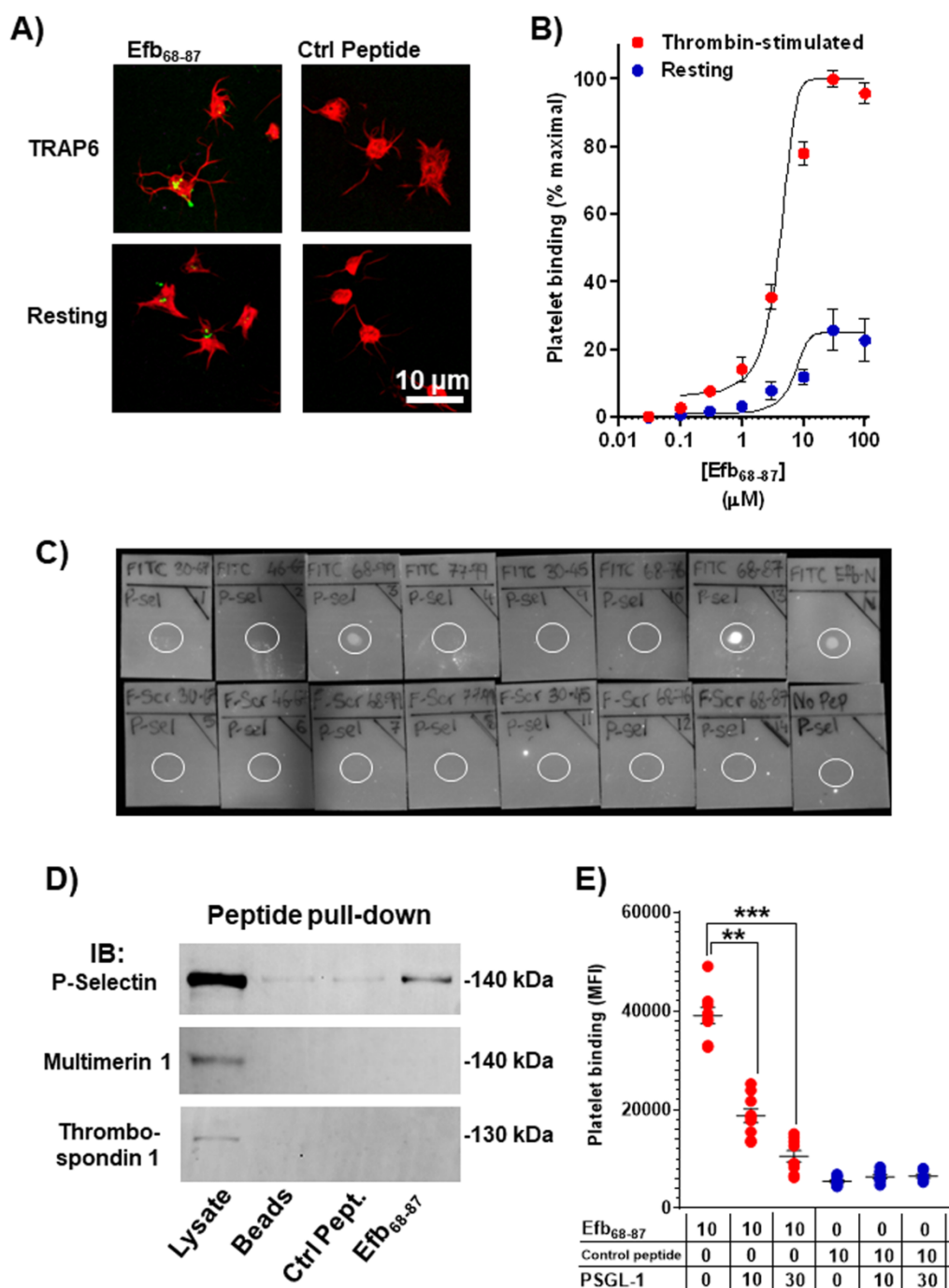


Figure 2

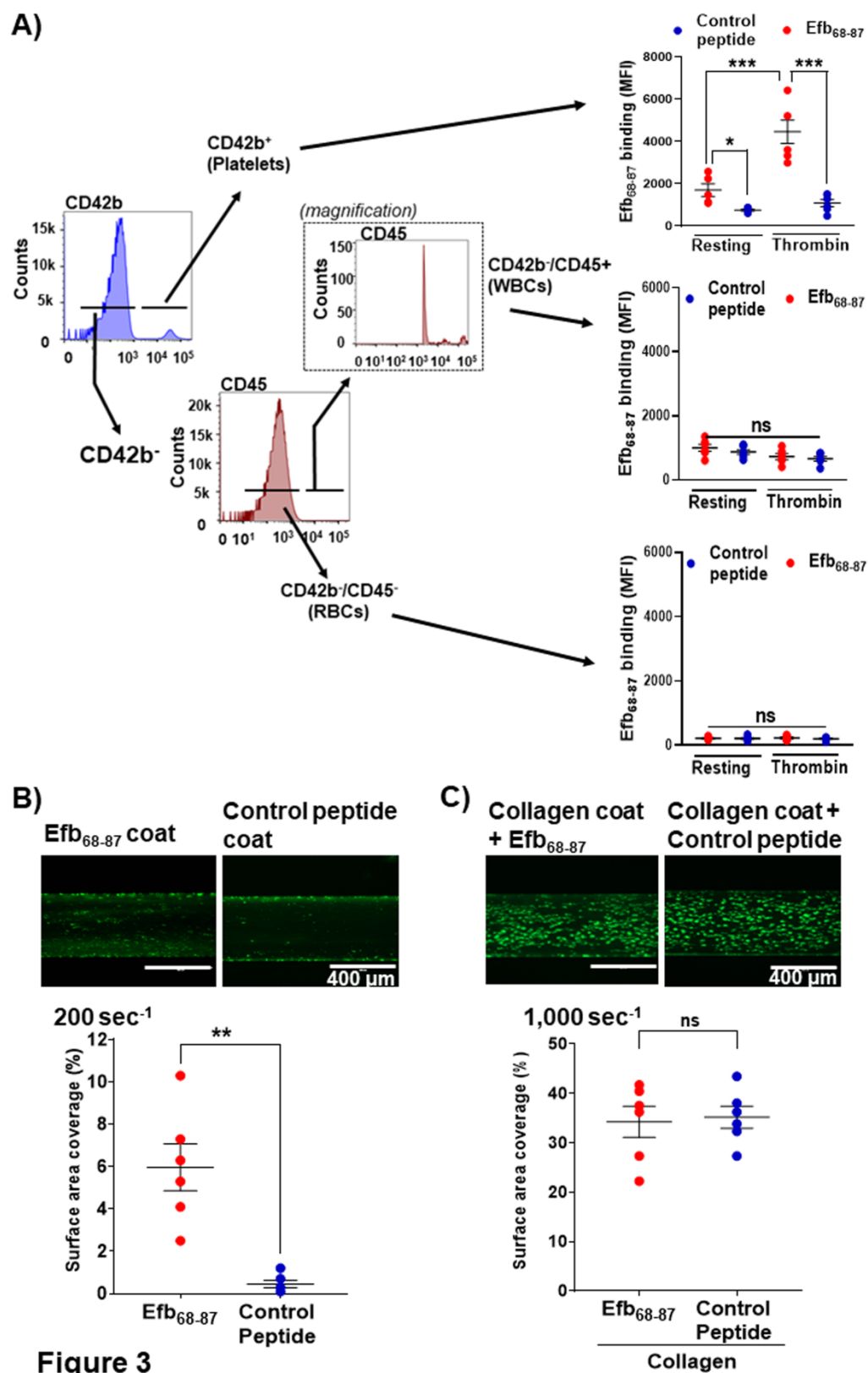


Figure 3

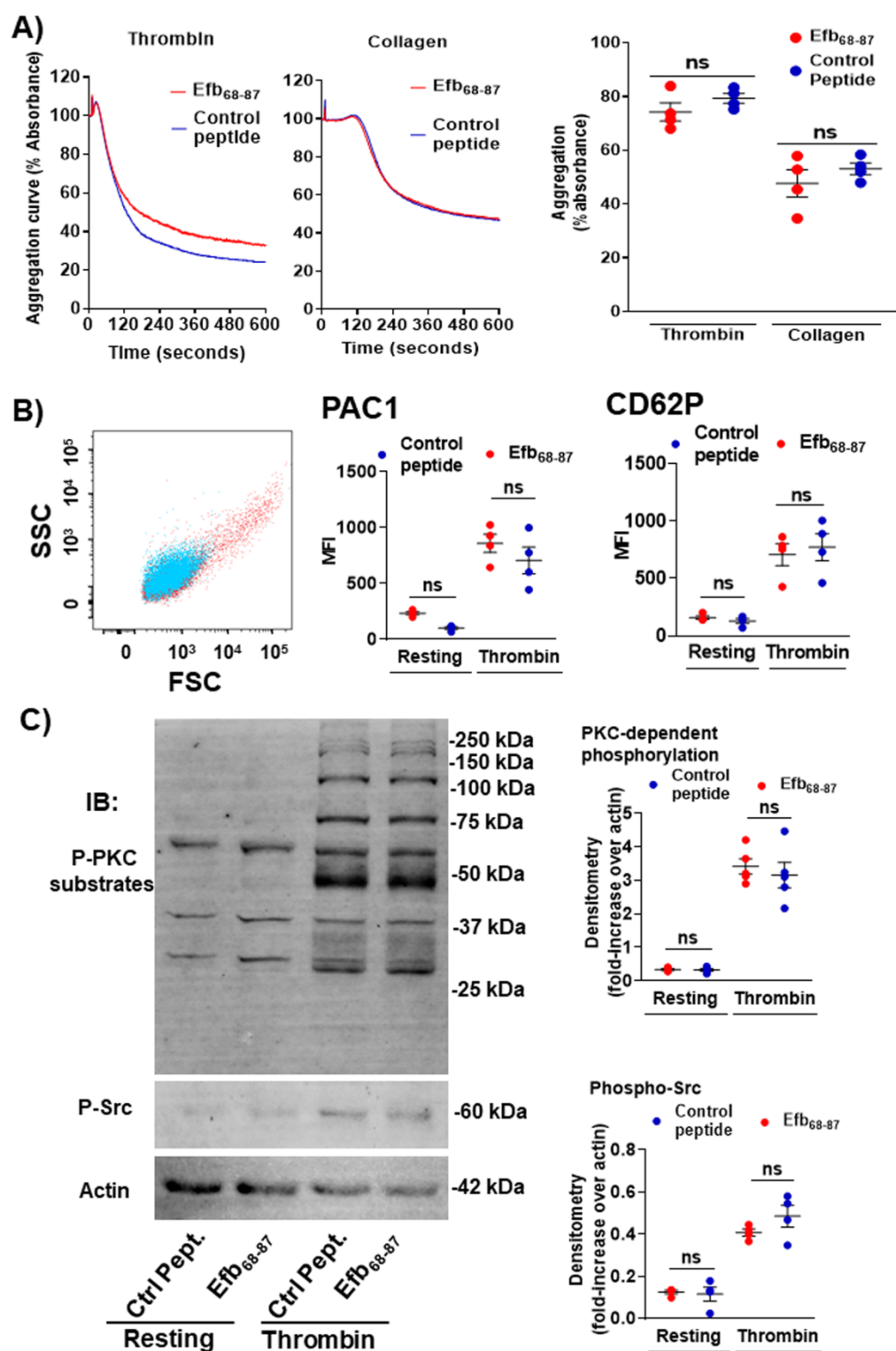


Figure 4

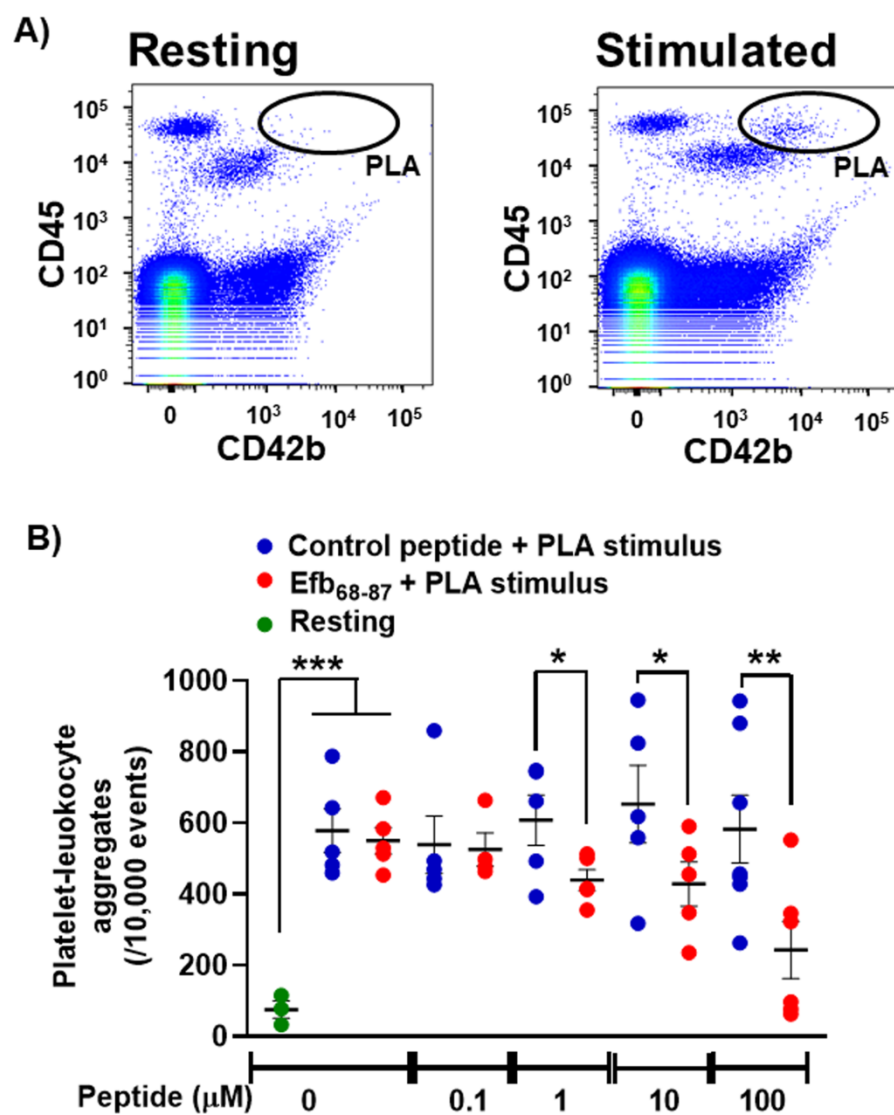


Figure 5

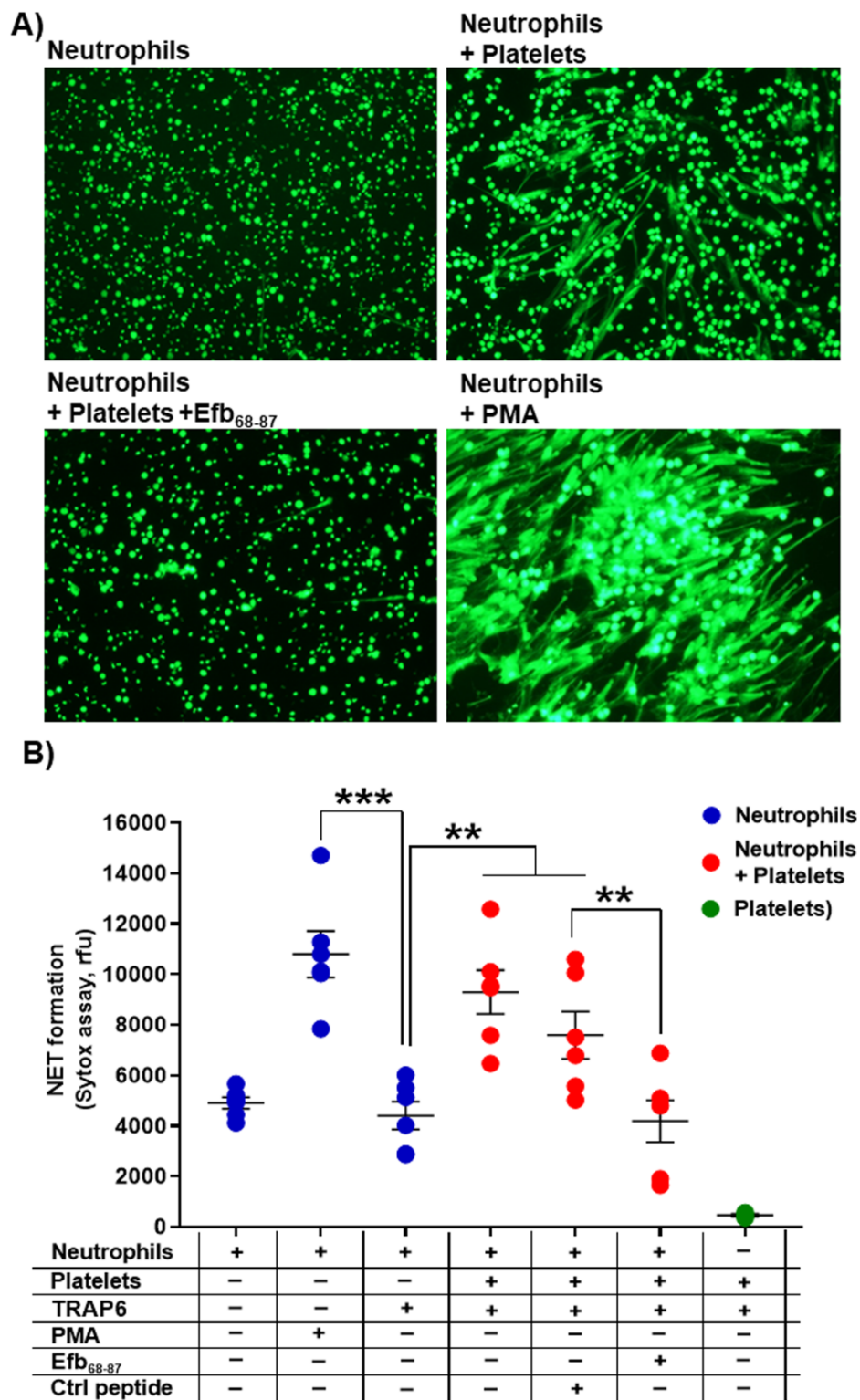


Figure 6